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ORIGINAL ARTICLE

Preparation and Evaluation of a New Lipopolysaccharide-based Conjugate as a Vaccine Candidate for Brucellosis

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Abstract
Objectives: Development of an efficacious vaccine against brucellosis has been a challenge for scientists for many years. At present, there is no licensed vaccine against human brucellosis. To overcome this problem, currently, antigenic determinants of *Brucella* cell wall such as Lipopolysaccharide (LPS) are considered as potential candidates to develop subunit vaccines.
Methods: In this study, *Brucella abortus* LPS was used for conjugation to *Neisseria meningitidis* serogroup B outer membrane vesicle (OMV) as carrier protein using carbodiimide and adipic acid–mediated coupling and linking, respectively. Groups of eight BALB/c mice were injected subcutaneously with 10 μg LPS alone, combined LPS + OMV and conjugated LPS–OMV on 0 days, 14 days, 28 days and 42 days. Anti-LPS IgG was measured in serum.
Results: The yield of LPS to OMV in LPS–OMV conjugate was 46.55%, on the basis of carbohydrate content. The ratio for LPS to OMV was 4.07. The LPS–OMV conjugate was the most immunogenic compound that stimulated following the first injection with increased IgG titer of ~5-fold and ~1.3-fold higher than that produced against LPS and LPS in noncovalent complex to OMV (LPS + OMV), respectively. The highest anti-LPS IgG titer was detected 2 weeks after the third injection (Day 42) of LPS–OMV conjugate. The conjugated compound elicited

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higher titers of IgG than LPS + OMV, that showed a 100–120-fold rise of anti-LPS IgG in mice.
Conclusion: These results indicate that our conjugated LPS–OMV can be used as a brucellosis vaccine, but further investigation is required.

1. Introduction

Brucellosis is one of the common bacterial zoonoses caused by organisms belonging to genus *Brucella*, a Gram-negative, non-spore-forming, facultative intracellular bacterium. Development of an efficacious vaccine against brucellosis has been a challenge for scientists for many years [1]. At present, there is no licensed vaccine against human brucellosis. To overcome this problem, currently, antigenic determinants of *Brucella* cell wall such as lipopolysaccharide (LPS) are considered as potential candidates for the development of subunit vaccines. Also, *Brucella abortus* LPS is considered as one of the most important antigens from the point of view of the primary targets of the innate immunity. The LPS of smooth strains of *Brucella* spp. comprise lipid A, fatty acids, a core region, and a polysaccharide O-side chain. The lipid A moiety alone is sufficient to activate the innate immune response; adaptive (antibody) responses are generated to the O antigen polysaccharide later in the course of infection [2].

Naturally occurring strains lacking LPS show reduced survival, therefore, LPS is considered to be a major virulence factor. Previous studies have clearly established that Smooth lipopolysaccharide is necessary for efficient intracellular survival and virulence of *Brucella melitensis*, *B. abortus and Brucella suis. B. abortus* S-LPS is 100 times less potent than that of *Escherichia coli* and *Salmonella* in inducing tumor necrosis factor γ produced by macrophages, as well as oxidative metabolism and lysozyme release by human neutrophils. This feature of S-LPS has been proposed to contribute to the survival of *B. abortus* within phagocytic cells. In addition, *Brucella* S-LPS is not susceptible to the actions of polycationic molecules, suggesting that smooth *Brucella* can resist the cationic bactericidal peptides of the phagocytes. S-LPS has also been found to confer antiphagocytic properties upon *Brucella* and is unable to activate the alternative pathway of the complement cascade [1–3].

It has been documented that polysaccharide (from bacterial capsule or LPS)–protein conjugates are usually immunogens in mice, rabbits and humans [4,5]. Many studies have shown that these conjugated vaccines can elicit humoral and cellular immunity against many human pathogens including *Neisseria meningitidis*, *Vibrio cholerae*, *Haemophilus influenzae*, *Shigella sonnei*, as well as *Brucella* spp. [4–7]. Covalent linkage of the polysaccharide, or fractions thereof, to immunogenic carriers (i.e., proteins) creates glycoconjugates that are T-dependent antigens and prime for boosting either with the glycoconjugate or the LPS. In contrast, polysaccharide or LPS–protein conjugate has been proven to be effective in several cases, and well-defined glycoconjugate vaccines have also been explored with a view to elicit discriminating immune responses [2,7,8]. Jacques et al showed the efficacy of *Brucella* O-polysaccharide–BSA conjugate in protection against *B. melitensis* H38 [9]. Other studies have been carried out to design subunit vaccines using other components and conjugated compounds such as porins and smooth lipopolysaccharide, recombinant ribosomal proteins and anti-OPS specific monoclonal antibodies [10–12].

The outer membrane vesicle (OMV) of *N. meningitidis* is among the newly studied components of microbial origin, which could be applied as a novel carrier. In addition, the potency of OMV as a carrier (conjugated to a hapten) is now proven. Overall, previous studies have shown that the predominant outer membrane proteins (PorA, PorB and RmpM) from *N. meningitidis* present in the Meningococci B Cuban vaccine had different capacities to prime the immune responses [1,13–16].

In the present study, we designed a subunit brucellosis vaccine composed of *B. abortus* S99 LPS with *N. meningitidis* serogroup B OMV as a covalent conjugate and then evaluated specific antibody response against the LPS of *B. abortus* S99.

2. Materials and methods

2.1. Bacterial strain, culture and fermentation conditions

B. abortus S99 was obtained from the Collection of Standard Bacteria, Pasteur Institute of Iran. This strain of *B. abortus* (biovar1) is smooth and CO_2 independent. It was cultured in slant *Brucella* agar medium (Merck, Germany) at 37 ± 1°C for 72 hours and then cultured in a 5-L flask containing 2 L *Brucella* broth (Merck) under aeration by a sparger at 37 ± 1°C for 72 hours to achieve the seed culture. Seed culture inoculated to the 60-L industrial fermenter (Novapaljas, contact-flow B.V, Netherlands) with a 40-L working volume [2]. The temperature and pH were adjusted to 37 ± 1°C and 6.4, respectively. Finally, after 60 hours, the fermentation process stopped by adding 10% phenol to the fermentation culture and biomass of *B. abortus* S99 harvested by centrifugation (1,19).

2.2. Extraction of B. abortus S99 LPS and chemical analysis

As described previously, *B. abortus* S99 LPS was extracted by an optimized method based on hot phenol−water and the extracted sample was chemically analyzed to define the content of LPS, 2-keto-3-deoxyoctonate, protein and nucleic acids (1,19).

2.3. Preparation of OMVs

OMVs were prepared as previously described [2,6,14]. *N. meningitidis* serogroup B strain CSBPI and G-245 cells were grown in 40 L modified Frantz medium in a fermenter for 24 hours at 36°C. The OMV was extracted from the cells by the method of Claassen et al [2,6,14].

2.3.1. Synthesis of LPS−OMV conjugates

LPS derivatives of *B. abortus* S99 were conjugated with *N. meningitidis* serogroup B OMV using adipic acid dihydrazide (ADH) as linker and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) as coupling reagent [8,14−17]. ADH was conjugated to LPS (96 mg) in 12 mL 287mM ADH suspension to form adipic hydrazide (AH)−LPS derivatives, using EDAC HCl and *N*-hydroxysulfosuccinimide. The resulting AH−LPS was finally coupled to OMV. Ten milligrams of OMV was reacted with 20 mg AH−LPS (10 mg/mL) using 0.05M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl. The reaction mixtures were maintained at pH 5.0−5.2 for 4 hours at 4°C, and the reactions were stopped by adjusting the pH to 7.0. The reaction mixtures were dialyzed against 0.9% NaCl for 2−3 days, centrifuged, and passed through a Sephacryl S-300 column (2.6 cm × 90 cm) in 0.9% NaCl. The peaks that contained both protein and carbohydrate were pooled and designated LPS−OMV. The conjugate was analyzed to determine the carbohydrate and protein contents using LPS and bovine serum albumin as standards [8,18].

2.4. Limulus amebocyte lysate assay

The LPS was tested to determine its endotoxin reactivity using a Limulus amebocyte lysate (LAL) Pyrogent Plus reagent kit (24 single test vials; Biowhittaker, Walkersville, MD, USA). The sensitivity of the LAL assay was 0.12 endotoxin unit (EU)/mL [13].

2.4.1. Immunization procedures

Groups of eight female BALB/c mice were injected subcutaneously four times at 2-week intervals with LPS−OMV conjugate (10 μg LPS in polysaccharide content), LPS + OMV (10 μg S-LPS plus 50 μg OMV) and LPS (10 μg polysaccharide) in 0.2 mL 0.9% NaCl. The immunized animals were bled prior to injection and 2 weeks after each injection [2].

2.4.2. Enzyme-linked immunosorbent assay

Specific antibody molecules produced against the extracted LPS of *B. abortus* were demonstrated by enzyme-linked immunosorbent assay (ELISA). Total specific IgG molecules, elicited against the S-LPS of *B. abortus* were detected. ELISA was performed in 96-well flat-bottom polystyrene microtiter plates. The wells were coated with the extracted S-LPS of *B. abortus* S99 at a concentration of 10 μg/mL in phosphate-buffered saline (PBS) azide (0.014M NaCl, 0.02% sodium azide, 0.01M sodium phosphate; pH 7.5) and incubated overnight at 37°C. The wells were washed three times with PBS azide. The wells were blocked by adding blocking buffer (1% casein in PBS−azide) and then incubated again at 37°C for 1 hour. Serial twofold dilutions of the sera were made on the plates. The plates were maintained at 25°C for 16 hours. Horseradish-peroxidase-labeled goat anti-mouse IgG was added to the wells at a concentration of 1 μg/mL and the plates were incubated at 25°C for 16 hours. Afterwards, the substrate of horseradish peroxidase (3,3′,5,5′-Tetramethylbenzidine) was added to the wells at a concentration of 1 mg/mL and the plates were incubated at room temperature for 30 minutes. Finally, stop solution (50 μL) was added to the wells and absorbance was measured at 405 nm using an automatic ELISA plate reader. The antibody titers are expressed in OD units and calculated by multiplying the reciprocal dilution of the serum by the OD at that dilution [2].

2.4.3. Statistical methods

Antibody titers of groups of mice were expressed as means ± standard deviations. The significance of differences in ELISA titers was determined by Student's *t* test.

3. Results

3.1. Fermentation process

The efficiency of the fermentation process to obtain *B. abortus* S99 biomass was satisfactory because the cell density and count of viable cells were 8.9% and 3.1×10^{11}, respectively. At the end of the fermentation process, microscopic evaluation to determine microbial content was carried out and the purity of resulting biomass was confirmed.

3.2. LPS characterization

LPS was isolated using the optimized hot phenol−water extraction method. The LPS concentration was 146 EU/mL. The LPS content of the extracted sample (measured by chromogenic LAL assay) was 108 EU/mL. The 2-keto-3-deoxyoctonate content (measured by Weisbach method) was 1.3%. In addition, the protein content of the sample (measured by picodrop) was < 2.0, and the nucleic acid content was $< 1\%$.

Table 1. Anti-*Brucella abortus* S99 LPS total IgG titer ± standard deviation, 2 weeks after the first, second and third injections.

Compound	Anti-*B. abortus* S99 LPS IgG titer, 2 wk after first injection	Anti-*B. abortus* S99 LPS IgG titer, 2 wk after second (first booster) injection	Anti-*B. abortus* S99 LPS IgG titer, 2 wk after third (second booster) injection
LPS	1,868 ± 106	6,828 ± 577	16,809 ± 1,276
LPS + OMV	7,398 ± 390	45,057 ± 2,779	91,993 ± 5,665
Conjugated LPS−OMV	9,386 ± 480	67,280 ± 2,539	112,978 ± 4,356
Control group (normal saline)	<3	<3	<3

3.3. Characterization of LPS−OMV conjugates

The ratio of the conjugates obtained in three experiments were similar, obtaining them with an average LPS/OMV ratio of 4.07. On the basis of carbohydrate, the yield of LPS to OMV in LPS−OMV conjugate was 46.55% content.

3.4. ELISA

Subcutaneous administration of *B. abortus* S99 LPS increased specific anti-LPS IgG titer in all the groups. Among the three injected compounds, LPS−OMV conjugate was the most immunogenic compound, promoting produced IgG titer following the first injection, which was fivefold and 1.3-fold higher than IgG titer elicited against LPS and LPS + OMV, respectively. The booster injections (especially the first booster) significantly increased the titer of anti-*B. abortus* S99 LPS IgG in all the groups ($p < 0.05$). The first and second booster injections of LPS−OMV conjugate caused 7.2-fold and 12-fold higher titers compared to the anti-LPS IgG titer induced after the first injection. The highest anti-LPS IgG titer was detected 2 weeks after the third injection (Day 42) of LPS−OMV conjugate (Table 1).

4. Discussion

We synthesized an LPS−OMV conjugate that was immunogenic in mice. This conjugate showed good antigenicity *in vitro* and elicited high levels of serum anti-LPS IgG in mice. The conjugated LPS elicited higher titers of anti-LPS IgG than LPS + OMV elicited, which showed a 1.2-fold rise of anti-LPS IgG in mice. These data are for the most part consistent with our previous studies with complex of *B. abortus* S99 LPS with *N. meningitidis* OMV. Our previous studies had demonstrated that LPS + OMV was immunogenic in mice and elicited a high level of anti-LPS IgG titers [2]. In this study, the conjugated LPS retained antigenic determinants when covalently bound to protein carriers. These data show that the use of a basic hydrolysis for the activation of LPS and the later use of EDAC for activation of OMV yields a conjugated compound that can elicit high levels of anti-LPS IgG in mice. At the same time, the data indicated that the carrier protein, in the form of an intact OMV, is an important factor for induction immunogenicity where the LPS component has to be protected.

Many studies have shown that polysaccharide (from bacterial capsule or LPS) or LPS−protein conjugates are usually effective immunogens in mice and rabbits as well as in humans. Regarding the use of LPS as a component for conjugates, many factors may result in differences of antibody response generated *in vivo*. These include the composition of conjugate, protein carrier, immunization routes, adjuvants, animal species, and detection methods [14]. Yu et al designed LPS-based conjugate vaccines and reported the serum bactericidal activity of these conjugates in rabbits and mice [4,5]. In meningococcal LPS conjugate studies, Jening et al showed that oligosaccharide-tetanus toxoid conjugates, elicited bactericidal antibodies in rabbits to the homologus strains [20−22]. In the same study, we synthesized the group A capsular polysaccharide−OMV of meningococci serogroup B conjugate and showed the serum bactericidal activity of this conjugate in rabbits [6,8].

As mentioned earlier, anti-*B. abortus* S99 LPS IgG titers exponentially and significantly increased in all groups compared to the control group. The high titers of anti-*B. abortus* S99 LPS IgG in comparison with the control group indicate the immunogenicity of *B. abortus* S99 LPS and the fidelity of our LPS extraction procedure which does not interfere with the natural and immunogenic structure of LPS. Furthermore, as mentioned above, during the process of conjugation, the antigenic determinants have been protected in all of the components (LPS + OMV).

In conclusion, conjugation of LPS to OMV was designed and the antigenicity of this conjugate was evaluated by ELISA. The conjugate elicited higher titers of IgG than LPS + OMV elicited, which showed a 120−165-fold rise of anti-LPS IgG in mice. These results indicate that our conjugated LPS, can be used as a brucellosis vaccine, but further investigation is required.

Conflicts of interest

Authors declare no conflict of interest.

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